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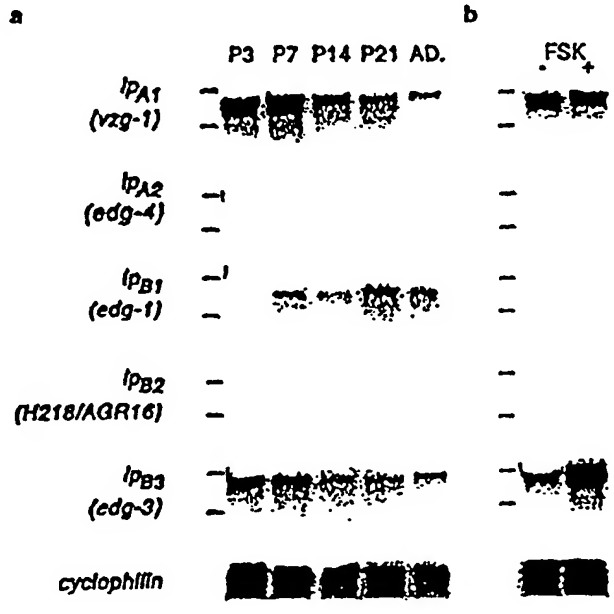
(54) Title: METHODS FOR PROMOTING SURVIVAL OF MYELIN PRODUCING CELLS

(57) Abstract

This invention is in the field of neurobiology, and relates particularly to methods useful for enhancing the survival of myelin producing cells, in particular Schwann cells and oligodendrocytes, and thereby to treating diseases of the nervous system involving loss of myelination or abberant myelination.



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METHODS FOR PROMOTING SURVIVAL OF MYELIN PRODUCING CELLS

FIELD OF THE INVENTION

This invention is in the field of neurobiology, and relates particularly to methods useful for enhancing the survival of myelin producing cells, in particular Schwann cells and oligodendrocytes, and thereby to treating diseases of the nervous system involving loss of myelination or abberant myelination.

BACKGROUND OF THE INVENTION

Schwann cells and oligodendrocytes are important glial cells that provide myelin sheathing around the axons of neurons. Schwann cells provide myelin sheathing around axons in the peripheral nervous system and oligodendrocytes provide myelin sheathing around axons in the central nervous system.

During peripheral nerve myelination in the early postnatal period, the matching of Schwann cells and axon numbers is regulated by Schwann cell apoptosis[5,6], resulting in the mature 1:1 relationship between axons and myelinating Schwann cells[10]. After this period of Schwann cell proliferation and myelination, Schwann cells are relatively inactive. However, upon injury Schwann cells will demyelinate around the injured axon and reenter the cell cycle thereby allowing production of remyelinating Schwann cells for the regenerated axon. Accordingly, it is believed that Schwann cells receive survival and differentiation signals from growing axons during peripheral nerve development and regeneration[10], based on studies of Schwann cells following nerve transection[6]. It has also be shown that oligodendrocytes will remyelinate after pathological lesions to the central nervous system. (Ludwin, S.K. Regeneration of myelin and oligodendrocytes in the central nervous system. *Progress in Brain Research*, V. 71, p. 469)

Myelin producing cells thus play an important role in the development, function, and regeneration of nerves. The implications of this from a therapeutic perspective have been addressed by Levi et al in J. Neuroscience, 1994, 14(3):1309, where the authors discuss the potential for cellular prostheses comprising human Schwann cells which could be transplanted into areas of damaged spinal cord. Accordingly, these authors outline the need for Schwann cell mitogens which can be used to allow full differention of these cells

ex vivo. WO 94/00140 describes the use of various factors for stimulating mitogenesis of glial cells including Schwann cells. Others have demonstrated that neuregulin is a potent mitogen for human Schwann cells in vitro.[5,6]

Agents that promote growth and survival of myelin producing cells can be useful for a variety of therapeutic purposes. Diseases and conditions of the nervous system that result from the deterioration of, or damage to, the myelin sheathing generated by myelin producing cells are numerous. Myelin may be lost as a primary event due to direct damage to the myelin sheath or as a secondary event as a result of damage to axons and neurons. Primary events include neurodegenerative diseases such as Multiple Sclerosis, human immunodeficiency MS-associated myelopathy, transverse myelopathy/myelitis, progresseive multifocal leukoencepholopahty, central pontine myelinolysis and lesions to the myelin sheathing (as described below for secondary events). Secondary events include a great variety of lesions to the axons or neurons caused by: physical injury; ischemia diseases, malignant diseases, infectious diseases (such as HIV, Lyme disease, tuberculosis, syphillis, or herpes), degenerative diseases (such as Parkinson's, Alzheimer's, Huntington's, ALS, optic neuritis, postinfectious enephalomyelitis, adrenoleukodystrophy and adrenomyeloneuropathy), nutritional diseases/ disorders (such as folic acid and Vitamin B12 deficiency, Wernicke disease); systemic diseases (such as diabetes, systemic lupus erthermatosos, carcinoma); and toxic substances (such as alcohol, lead, ethidium bromide); and iatorgenic processes such as drug interactions, radiation treatment or neurosurgery.

SUMMARY OF THE INVENTION

The present invention provides a method for promoting the survival of myelin producing cells, in particualar Schwann cells and oligodendrocytes. Other embodiments of the present invention are directed to therapeutic methods, utilities, and other related uses.

One aspect of the present invention is a method for promoting the survival of myelin producing cells comprising treating myelin producing cells with an effective amount of an LPA receptor agonist. In particular, there is provided a method for

promoting the survival of Schwann cells comprising the step of treating the Schwann cells with an effective amount of an LP_{A1}/VZG-1/edg-2 receptor agonist.

Another aspect of the invention is a method for enhancing the development or regeneration of myelin by promoting the survival of myelin producing cells comprising treating myelin producing cells with an effective amount of an LPA receptor agonist. In a preferred embodiment, the myelin producing cells are Schwann cells and the LPA receptor agonist is an LP_{A1}/VZG-1/edg-2 receptor agonist.

Yet another aspect of the invention is a method for promoting survival of endogenous myelin producing cells in a subject comprising delivering to the subject an effective amount of an LPA receptor agonist. In a particular embodiment, the myelin producing cells are Schwann cells and the LPA receptor agonist is an LP_{A1}/VZG-1/edg-2 receptor agonist.

Another aspect of the invention is a pharmaceutical composition useful for treating a neurological disorder involving a loss of myleination comprising delivering to the subject an effective amount of an LPA receptor agonist, in particular, an effective amount of a LP_{A1}/VZG-1/edg-2 receptor agonist.

Another aspect of the invention, is a method for treating a subject suffering from a neurological disorder involving a loss of myelination comprising delivering to the subject an effective amount of an LPA receptor agonist, in particular, an effective amount of a LP_{A1}/VZG-1/edg-2 receptor agonist.

Another aspect of the invention is a serum-free medium suitable for culturing myelin producing cells, in particular Schwann cells comprising an effective amount of an LPA receptor agonist, preferably a LP_{A1}/VZG-1/edg-2 receptor agonist, to promote survival of the myelin producing cells and suitable cell culturing excipients necessary for cell viability.

Another aspect of the invention is use of serum-free medium or LPA agonist-supplemented medium for expanding myelinating precursor cells that could be used for

transplantation to repair demyelinated lesions produced by any of the aforementioned disesases or processes.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention are described hereinafter with reference to the accompanying drawings as described below.

Figure 1. Members of the lysophospholipid (*lp*) receptor gene family are expressed by Schwann cells *in vivo* and *in vitro*. Northern blots of 10 μg total RNA from sciatic nerve (**a**) at various postnatal (P) ages to adult (AD.), or of 15 μg total RNA from cultured neonatal (P3) sciatic nerve Schwann cells (**b**) grown with (+) and without (-) forskolin (FSK), were probed for transcripts encoding five members of the LP GPCR family[4]. The LPA receptor lp_{A1}/*vzg-1* is expressed at high levels by Schwann cells both *in vivo*, especially in the first 2 postnatal weeks, and *in vitro* with and without FSK, which induces a more differentiated Schwann cell phenotype[10]. Expression of a related receptor gene (lp_{A2}/*edg-4*) recently reported to mediate LPA responses [25] was undetectable in Schwann cells either *in vivo* or *in vitro*. Of three genes (lp_{B1-3}) which encode S1P receptors [12], only lp_{B3} is prominently expressed by neonatal Schwann cells *in vitro*, although both lp_{B1} and lp_{B3} are expressed at various ages *in vivo*(the absence of lp_{B1}/*edg-1* expression *in vitro* reflects the very low expression in the sciatic nerve at P3, the age at which cultures were made). A *cyclophilin* probing is shown as a loading and transfer control. Upper marker is 4.4 kb and lower marker is 2.4 kb in each panel.

Figure 2. LPA is a specific, potent survival factor for Schwann cells following serum withdrawal. **a**, photomicrographs of control and LPA (1 μM)-treated SC cultures 48 h after serum withdrawal, either prior to fixation (phase contrast, top row), or after fixation and staining for ISEL+, BrdU-immunofluorescence, and/or DAPI nuclear counterstaining (the DAPI and ISEL+ panels represent the same field of view; the phase contrast and BrdU panels are from parallel cultures). Total cell number is greater in LPA-treated cultures, while the number of apoptotic cells detected morphologically or by ISEL+ labelling is decreased. In contrast, LPA treatment does not affect BrdU incorporation. Scale bars, 30 μM. **b**, LPA significantly decreases apoptosis as detected by ISEL+ at doses as low as 10 nM. Numbers are expressed as percent of control ISEL+-

labelled cell numbers. *p < 0.05; **p < 0.0001 (vs. control). **inset**, northern blot analysis of 7 μg total RNA shows that *lp$_{A1}$/vzg-1* expression is maintained following serum withdrawal. **c**, BrdU incorporation is not affected by LPA-treatment following serum withdrawal, though cells are capable of proliferation following addition of 10% fetal calf serum (FCS). *p < 0.0001 (vs. control). **d**, The survival-promoting effect of LPA is also evidenced by increased maintenance of cell number following 48 h-serum withdrawal. *p < 0.0001 (vs. control). **e**, The lysosphingolipid S1P does not promote Schwann cell survival, indicating a specific effect of LPA (S1P vs. control, p > 0.3). *p < 0.0001 (vs. control). **f**, LPA (1 μM) is as effective in promoting Schwann cell survival as a maximal dose (100ng/ml, determined in pilot experiments) of a highly active, truncated form of neuregulin (NRG__[21]), a proven promoter of Schwann cell survival[5,6]. LPA and NRG_ do not act synergistically when added together at these maximal doses, and only slightly but not significantly so at lower doses (not shown), suggesting that their signalling pathways share a common downstream effector. *p < 0.01 (vs. control). LPA, NRG_, and LPA + NRG_ treatments were not significantly different from each other. Data presented in **a** - **f** are means (with s.e.m. bars) of 3-5 experiments performed in duplicate. p values are from ANOVA with Fisher's PLSD post-hoc analyses.

Figure 3. Promotion of Schwann cell survival by LPA involves G_i and the PI3K/Akt pathway. **a**, promotion of Schwann cell survival by LPA ("----" lane) is significantly reduced by pretreatment of cells with pertussis toxin (PTX), indicating the involvement of G_i, and is completely blocked by wortmannin(Wort.) and LY294002 (LY), indicating its dependence on PI3K. Data presented are means (with s.e.m. bars) of 3-4 experiments performed in duplicate. *p < 0.0001 (vs. control), by t-test with matched control. All other means were not significantly different from their matched controls (i.e., pharmacological inhibitor alone; p values between 0.1 and 0.6). **b, c**, western blots probed with antibodies specific for the Ser473-phosphorylated form of Akt (_-phospho-Akt), with parallel loading control blots probed with antibodies detecting all Akt (_-Akt). Treatment of Schwann cells with LPA (1 μM) induces a transient (< 1h, **b**) increase in Akt phosphorylation at a site (Ser473) required for its activation[8]. This LPA-induced increase in Akt phosphorylation is blocked by pretreatment of Schwann cells with Wort. or LY, but not with the MAP kinase pathway inhibitor PD98059 (PD, **c**), consistent with data showing that PI3K activation is upstream of Akt activation[8] kDa, kilodaltons.

Figure 4. Overexpression of the LPA receptor LP_{A1}/VZG-1 potentiates Schwann cell survival. **a**, photomicrographs of Schwann cells transfected with pFLAG/VZG-1 (encoding a FLAG-epitope tagged LP_{A1}/VZG-1 receptor) or with pFLAG/BAP (encoding a FLAG-epitope tagged bacterial alkaline phosphatase control protein), double-labelled for _-FLAG immunofluorescence (red) and a fluorescent ISEL technique (green) to detect apoptotic cells. Double-labelled, apoptotic transfected cells (arrows), as well as healthy transfected cells (arrowheads), are clearly identifiable. Scale bars, 30 μM. **b**, overexpression of epitope-tagged LP_{A1}/VZG-1 significantly potentiates SC survival (vs. transfection control) both with and without a sub-maximal (0.1 μM) dose of LPA. The effect of 1 μM LPA was also potentiated, but this failed to reach significance ($p = 0.19$). Data (% apoptotic transfected cells) are presented as means (with s.e.m. bars) of 3 experiments performed in triplicate. *$p < 0.005$ (vs. matched pFLAG/BAP transfection control condition) by ANOVA (significant main effects of LPA treatment and plasmid transfection conditions) and Fisher's PLSD post-hoc analyses.

Figure 5. Decreased LPA-responsiveness and increased apoptosis in Null mice for the LP_{A1}/VZG-1/ edg-2 receptor. **a**, Weak response of lp_{A1} (-/-) relative to lp_{A1} (+/+) Schwann cells following100 nM LPA treatment. Wild-type cells respond with process retraction and cell spreading (arrowheads; f-actin staining with TRITC-phalloidin).Schwann cells were isolated from the sciatic nerves of wild-type (control) animals or nerves from surviving lpA1 homozyous null (-/-) animals. The resulting cells were analyzed in primary cell culture. Bar = 40 μm. **b**, Quantified SC lysophospholipid responsiveness. Bars are means ± s.e.m. *$p < 0.03$. LPA concentrations used (1 μM) provide maximal responsiveness in this assay (JAW, NF, JC; in preparation). **c**, RT-PCR detection of lp_{A2} and lpB1 in both intact P7 sciatic nerves and neonatal Schwann cell cultures from lp_{A1}(-/-) mice. **d**, Increased apoptosis and decreased Schwann cell number in adult lp_{A1} (-/-) sciatic nerves. Sections were stained for apoptotic DNA fragmentation (using ISEL+) or nuclei (using DAPI). Bar = 40 μm. **e**, Apoptosis increases in lp_{A1} (-/-) sciatic nerves. Bars are means ± s.e.m. (*$p < 0.005$; unpaired t-test). **f**. Electron microscopic confirmation of Schwann cell apoptosis in lp_{A1} (-/-) sciatic nerves. Note

nuclear chromatin condensation (arrows) and cytoplasmic fragmentation characterisitic of apoptosis in adjacent to myelin sheaths surrounding an axon. Bar = 1 μm (top); 0.6 μm (bottom). ax, axon; my, myelin sheath.

DEFINITIONS

In general, the following words or phrases have the following meanings:

"Administer" means the process by which the desired agent or progenitor of the agent, e.g. prodrug, is delivered to the subject, such that said agent is contacted with the organs, tissues or cells of the subject in need of treatment. Administration can be made by any accepted systemic or local route.

"Cell culturing excipients" are those excipients necessary for sustaining the viability of cells in a cell culture environment.

"Effective amount" means that amount of an active agent when administered to a subject in need thereof, is sufficient to bring about the desired effect. For example, in the present invention an effective amount is such amount when administered to a subject would promote survival of myelin producing cells, such as, Schwann cells or oligodendrocytes.

"*Ex vivo*" means where cells are taken outside of a living organism to undergo a process or processing and then re-introduced back to a living organism.

"*In vitro*" means outside of a living organism; pertaining to conditions or to experiments with a perfused organ, a tissue slice, cells in tissue culture, a homogenate, a crude extract or a subcellular fraction.

"LPA receptors" are edg receptors that mediate physiological or pathophysiological effects of LPA by physical interactions between said receptor and LPA

"LPA receptor agonists" are agonists which activate LPA receptors and include LPA and other compounds (as exemplified below) which activate the LPA receptors as can be determined in a number of assays. LPA receptor agonists can be determined by employing the assay set out in Hecht et al. *Journal of Cell Biology* 135 1071-1083 (1996) (Reference 2), incorporated herein by reference, and which encompasses the use of ^{3}H-LPA bound specifically to cells that overexpress or heterologously express the LPA receptor (see also Fukushima et al., *Proc. Natl. Aca. Sci.* USA 95: 6151-6156, 1998, incorporated herein by reference. A single receptor encoded by *vzg-1/lp$_{a1}$/edg 2* couples to G-proteins and mediates multiple cellular responses to LPA. *Proc. Natl. Aca. Sci.* USA 95, 6151-6156. Other assays include the use of cell rounding or stress fiber formation in cells that do not express the receptor; once the receptor is heterologously expressed, these cells will then either round (in the case of the neuroblastoma cell line B103) or form stress fibers (for the liver cell line RH7777) when exposed to LPA at nM concentrations but not after exposure to related ligands. Another assay is to measure cAMP levels, since LPA activating its receptor produces a decrease in cAMP by activation of the heterotrimeric G-protein G_i. Another way is to assay the proximal event in G-protein coupling through the use of ^{35}S-GTPγS labelling of G-proteins that is dependent on the presence of an LPA receptor and LPA stimulation. Each of these methods is standard and noted in the citations.

"myelin producing cells" are cells that provide myelin and myelin sheathing, typically around the axons or neurons; Schwann cells and oligodendrocytes

"neurological disorder" is any disease, disorder, injury or iatrogenic process of or in the nervous system that results from , or in, the deterioration of, or damage to, the myelin sheathing and/or non-myelinated parts of the myelin producing cells that affects the process, maintenance or normal turnover/metabolism involved in myelination by myelin producing cells, including (but not limited to): neurodegenerative diseases of myelin such as: Multiple Sclerosis; human immunodeficiency MS-associated myelopathy; transverse myelopathy/myelitis; progresseive multifocl leukoencephalopathy; central pontine myelinolysis; and lesions to the nervous system caused by: physical injury; ischemic diseases; malignancy; infectious diseases (such as HIV, Lyme disease, tuberculosis, syphillis, or herpes); degenerative diseases (such as

Parkinson's, Alzheimer's, Huntington's ALS, optic neuritis, postinfectious enephalomyelitis, adrenoleukodystrophy and adrenomyeloneuropathy); nutritional diseases/ disorders (such as folic acid and Vitamin B12 deficiency, Wernicke disease); systemic diseases (such as diabetes, systemic lupus erthermatosos, carcinoma); and, toxic substances (such as alcohol, lead, ethidium bromide; and iatrogenic processes such as drug interactions, radiation treatment or neurosurgery.

"Pharmaceutically acceptable excipients" refers to ingredients that are combined with an active agent to form a pharmaceutical composition. Typically pharmaceutically acceptable excipients include, but are not limited to, a conventional pharmaceutical carrier, other medicinal agents, pharmaceutical agents, carriers, adjuvants, etc. Carriers can be selected from the various oils, including those of petroleum, animal, vegetable or synthetic origin (eg. peanut oil, soybean oil, mineral oil, sesame oil, and the like). Water, saline, aqueous dextrose, and glycols are preferred liquid carriers, particularly for injectable sollutions. In addition, suitable pharmaceutical carriers include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. Preferably pharmaceutical compositions will take the form of a capsule, pill or tablet, and thus pharmaceutically acceptable excipients will include but are not limited to a diluent such as lactose, sucrose, dicalcium phosphate, and the like; a disintegrant such as starch or derivative thereof; a lubricant such as magnesium stearate and the like; and a binder such as a starch, polyvinylpyrrolidone, gum acacia, gelatin, cellulose and derivatives thereof, and the like. For oral administration to infants, a liquid formulation (such as a syrup or suspension) is preferred. Other suitable pharmaceutical carriers and their formulations are descreibed in "*Remington's Pharmaceutical Sciences*" by E.W. Martin

"promoting survival of myelin producing cells" is increasing the viable lifespan of the myelin producing cells

"serum-free cell growth medium," "serum-free medium" or "serum-free composition" refers to a medium/compositions that are essentially free of serum from any

mammailan source e.g. contains lcss than about 0.5% serum, preferably less than about 0.1%, and most preferably less than 0-0.1%

"VZG-1" is a G-protein coupled receptor for LPA termed ventricular zone gene-1, alternatively this receptor is aslo designated LP_{A1} or edg-2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to agonists of LPA receptors to regulate myelination by promoting survival of myelin producing cells. In particular, it is demonstrated that agonists of the LPA receptors, including LP_{A1}/VZG-1/edg 2, have the potential to promote Schwann cell survival by activation of such LPA receptors. In view of the expression of the LP_{A1}/VZG-1/edg 2 receptor by oligodendrocytes[3] it is believed that a similar biological response to LPA and agonists of LPA receptors, particularly agonists of LP_{A1}/VZG-1/edg 2 receptors, would be observed in oligodendrocytes during central nervous system myelination in development and following injury. Furthermore, by promoting the survival of myelin producing cells the regeneration and development of myelin will be enhanced by the virtue of the viability of such surviving myelin producing cells. Accordingly, it is believed that agonists of LPA receptors, in particular agonists of LP_{A1}/VZG-1/ edg-2 receptors, have therapeutic relevance to a range of disorders in which myelination is perturbed.

LPA is present in serum containing Schwann cells in the micromolar range[11], making it a candidate molecule for mediating the Schwann cell survival-promoting effects of serum. The LPA receptor gene *lp_{A1}/vzg-1*[2] is well placed to mediate such effects since it is expressed by sciatic nerve Schwann cells *in vivo* throughout the postnatal period, with highest expression in the first week (Fig. 1a and 5c). Similarly, *lp_{A1}/vzg-1* is expressed at comparable levels by neonatal Schwann cells *in vitro* (Fig. 1b). Conversely, as expected, the null mice for the LP_{A1}/VZG-1/ edg-2 receptor show no expression in the nerve or Schwann cell cultures. (Fig. 5c) Other members of the recently identified lysophospholipid (LP) GPCR family, including identified receptors for the structurally and functionally related lipid sphingosine-1-phosphate (S1P)[12], are also expressed by Schwann cells (Fig. 1). These data indicate the potential action of receptor-mediated lysophospholipid signaling pathways during Schwann cell maturation.

It is demonstrated herein that LPA activates a pharmacologically defined signaling pathway in Schwann cells, involving G_i and phosphoinositide 3-kinase (PI3K), and induces phosphorylation of Akt, a kinase that mediates PI3K-dependent survival[7,8]. It is also demonstrated that overexpression of epitope-tagged LP_{A1}/VZG-1/edg 2 increased LPA-dependent Schwann cell survival. Furthermore, it is demonstrated that the null mice for LP_{A1}/VZG-1/ edg-2 receptor have decreased Schwann cell survival as compared to wild-type mice. Accordingly, the LPA receptors, particularly, the LP_{A1}/VZG-1/edg 2 receptor, and activation thereof, promotes Schwann cell survival.

Pharmaceutical Compositions

The pharmaceutical compositions of this invention are useful in promoting the survival of myelin producing cells, in particular, Schwann cells and oligodendrocytes. Therefore, these compositions are particularly useful for preventing, treating or ameliorating diseases and/or conditions of the nervous system that result from the deterioration of, or damage to, the myelin sheathing generated by myelin producing cells. Myelin may be lost as a primary event due to direct damage to the myelin or as a secondary event as a result of damage to axons and neurons. Primary events include neurodegenerative diseases such as Multiple Sclerosis, human immunodeficiency MS-associated myelopathy, transverse myelopathy, progresseive multifocal leukoencepholopathy; central pontine myelinolysis; and lesions to the nervous system (as described below for secondary events). Secondary events include a great variety of lesions to the axons or neurons caused by physical injury; ischemia diseases; malignant diseases; infectious diseases (such as HIV, Lyme disease, tuberculosis, syphillis, or herpes); degenerative diseases (such as Parkinson's, Alzheimer's, Huntington's ALS, optic neuritis, postinfectious enephalomyelitis, adrenoleukodystrophy and adrenomyeloneuropathy); nutritional diseases/ disorders (such as folic acid and Vitamin B12 deficiency, Wernicke disease); systemic diseases (such as diabetes, systemic lupus erthermatosos, carcinoma); and, toxic substances (such as alcohol, lead, ethidium bromide; and iatrogenic processes such as drug interactions, radiation treatment or neurosurgery.

Administration

The pharmaceutical composition of the present invention comprises an effective amount of a LPA receptor agonist, and pharmaceutically acceptable excipients. Those of ordinary skill in the art recognize that an "effective amount" means that amount of an active agent when administered to a subject in need thereof, is sufficient to bring about the desired effect. For example, in the present invention an effective amount is such amount when administered to a subject would promote survival of myelin producing cells, such as, Schwann cells or oligodendrocytes. Administration of the active compounds and salts described herein can be via any of the accepted modes of administration for active agents that serve similar utilities.

The level of active agent in a formulation can be within the full range employed by those skilled in the art, e.g. from about 0.01 percent weight (%wt to about 99.99%wt of the drug based on the total formulation and about 0.01% to 99.99%wt excipient.

Generally, an acceptable daily dose is of about 0.001 to 50 mg per kilogram body weight of the recipient per day, preferably about 0.05 to 10 mg per kilogram body weight per day. Thus, for administration to a 70 kg person, the dosage range would be about 0.07 mg to 3.5 g per day, preferably about 3.5 mg to 1.75 g per day, and most preferably about 0.7 mg to 0.7 g per day depending upon the individuals and disease state being treated. Such use optimization is well within the ambit of those of ordinary skill in the art.

If desired, the pharmaceutical composition to be administered may also contain minor amounts of non-toxic auxillary substances such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc.

EXAMPLES

The following examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

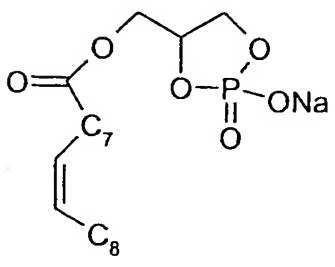
EXAMPLE 1

PREPARATION OF SCHWANN CELLS: REAGENTS AND PHARMACOLOGICAL TREATMENTS

Lyophilized LPA (1-Oleoyl-2-Hydroxy-sn-Glycero-3-Phosphate; Avanti Polar Lipids) was resuspended in 1% fatty acid-free (FAF) BSA (Sigma), and diluted for experiments in the same solution. Sphingosine-1-phosphate (S1P) (Biomol) was dissolved in methanol, aliquoted and lyophilized, and resuspended for experiments in 0.01% FAF BSA. S1P activity was confirmed in an independent morphological assay using the B103 neuroblastoma cell line[3]. Wortmannin, LY294002, and PD98059 (Calbiochem) were dissolved in DMSO at 10 mM, 50 mM or 100 mM, respectively, and diluted in PBS for experiments. Pharmacological inhibitors were added at the time of serum withdrawal and LPA addition for ISEL+ experiments, or at the time of serum withdrawal and 2 hours before LPA treatment for Akt experiments. For ISEL+ experiments, pertussis toxin (Calbiochem) was added to cultures 18h prior to serum withdrawal, at the time of serum withdrawal and LPA addition, and again 24 h later. Truncated GST-NRG__ (encompassing the EGF-like motif; see [25] for sequence) was the generous gift of Dr. Cary Lai, Scripps Research Institute, La Jolla, CA.

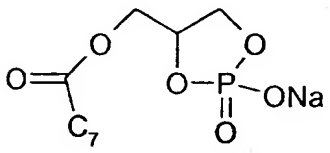
LP_{A1}/VZG-1/EDG-2 RECEPTOR AGONISTS

1.



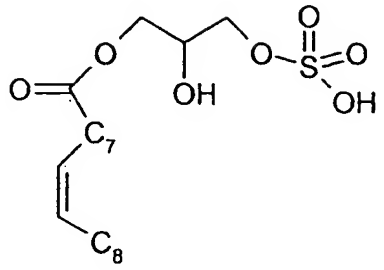
$P(O)Cl_3$ (0.19mL) was added to a mixture of monoolein (0.6464 g), EtOAc (15 mL), and Et_3N (2.92 mL). The mixture was stirred at 60 C for 2 h and then the mixture was cooled and the triethylamine hydrochloride was filtered off. Sodium hydroxide (20 mL of 0.2 M) was added and the biphasic solution was stirred for 12 h. EtOAc (200 mL) was added along with brine (200 mL). The layers were separated and the organic layer was evaporated and pumped to dryness. Thin Layer chromatography (silica gel, $CHCl_3$ 60, MeOH 30, HOAc 1, H_2O 1) of the product was compared to LPA (Sigma). The product ran with an Rf of ~0.7 and the LPA (Sigma) ran at 0.2. On treatment with acid the product ran at the same Rf as LPA (Sigma).

2.



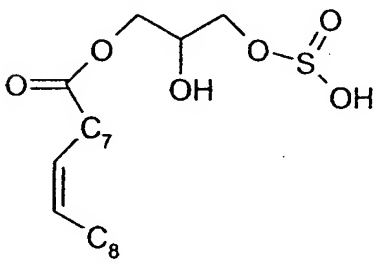
$P(O)Cl_3$ (0.47 mL) was added to a mixture of monocaprylin (1 g), EtOAc (30 mL), and Et_3N (3.19 mL). The mixture was stirred at 60 C for 1.5 h and then the mixture was cooled and the triethylamine hydrochloride was filtered off. Sodium hydroxide (45 mL of 0.2 M) was added and the biphasic solution was stirred for 12 h. EtOAc (200 mL) was added along with brine (200 mL). The layers were separated and the organic layer was evaporated and pumped to dryness. Thin Layer chromatography (silica gel, $CHCl_3$ 60, MeOH 30, HOAc 1, H_2O 1) of the product was compared to LPA (Sigma). The product ran with an Rf of ~0.7 and the LPA (Sigma) ran at 0.2. On treatment with acid the product ran at the same Rf as LPA (Sigma).

3.



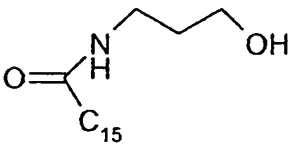
$ClS(O)_2OH$ (51L) was added to a solution of monoolein (250 mg), CH_2Cl_2 (10 mL) and pyridine (0.28 mL). The mixture was stirred at room temperature for 1 h. CH_2Cl_2 (100 mL) was added along with H_2O (50 mL). The mixture was separated and the pyridine was extracted using $CuSO_4$ (10%, 40 mL). The organic phase was dried, and evaporated and the residue was purified by flash chromatography (silica gel, 20% MeOH in $CHCl_3$) to give the product (50mg, 16 % yield).

4.



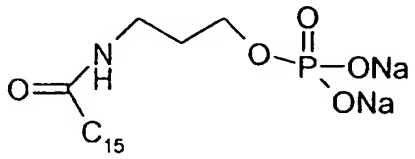
$S(O)Cl_2$ (0.11 mL) was added to a solution of monoolein (400 mg), EtOAc (10 mL) and Et_3N (0.78 mL). The mixture was stirred at room temperature for 10 min. The mixture was cooled and the Et_3NHCl was filtered off. NaOH (0.2 M, 13 mL) was added and the mixture was stirred for 12 h. The layers were separated and the water layer was rotovaped to dryness.

5.



(Intermediate)

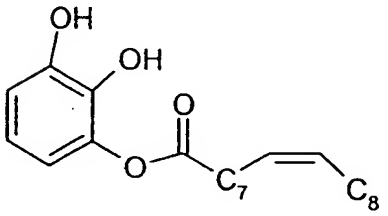
Lauroyl chloride (0.55 mL) was added to a cooled (-78 C) solution of 1-propanol 3-amine (0.14 mL), $CHCl_3$ (10 mL), and Et_3N (1.27 mL). The mixture was warmed to room temperature and stirred for 1 h. $CHCl_3$ (100 mL) was added and washed with H_2O (2 times, 50 mL). The organic phase was dried, and evaporated and the residue was purified by flash chromatography (silica gel, 5 % MeOH in $CHCl_3$) to give the product (0.35 g, 61 % yield).



(agonist)

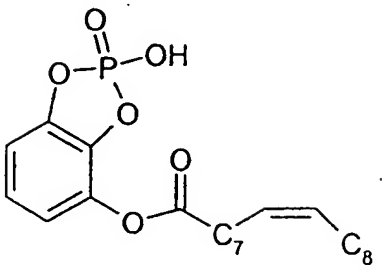
$P(O)Cl_3$ (90 L) was added to a solution of Lauryl 1-propanol 3-amide (50 mg), $CHCl_3$ (5 mL) and Et_3N (266 L). The mixture was stirred at room temperature for 1 h. The mixture was cooled and the Et_3NHCl was filtered off. NaOH (0.2 M, 2 mL) was added and the mixture was stirred and shaken vigorously. The layers were separated and the water layer was washed with $CHCl_3$ (3 times, 30mL). The organic layer was evaporated and the product was obtained.

6.



(Intermediate)

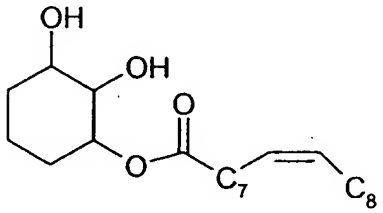
Oleoyl chloride (1.31 mL) was added to a cooled (0 C) solution of pyrogallol (1 g), CH_2Cl_2 (20 mL), and pyridine (0.96 mL). The mixture was stirred and warmed to room temperature over 2 h. CH_2Cl_2 (100 mL) was added and the organic phase was washed with H_2O (2 times, 50 mL). The organic phase was dried, and evaporated and the residue was purified by flash chromatography (silica gel, gradient 10% to 20% EtOAc in Hexane) to give primarily the 1 substituted product (0.8646 g, 28% yield).



(Agonist)

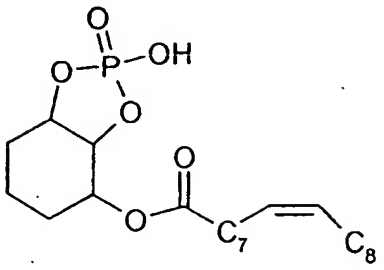
$P(O)Cl_3$ (0.13 mL) was added to a mixture of 1 and 2 substituted pyrogallol oleate (0.5 g), EtOAc (10 mL) and Et_3N (0.89 mL). The mixture was stirred at 60 C for 2 h. The mixture was cooled and the Et_3NHCl was filtered off. NaOH (0.2 M, 15 mL) was added and the mixture was stirred for 2 h. EtOAc (100 mL) was added and the organic layer was washed with H_2O (2 times 30 mL). The organic layer was evaporated to give the product.

7.



(Intermediate)

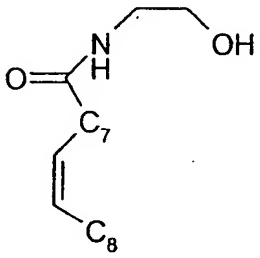
Oleoylchloride (2.49 mL) was added to solution of 1,2,3-cyclohexanetriol (2 g), CH_2Cl_2 (30 mL), and pyridine (1.84 mL). The mixture was stirred at room temperature for 2 h. CH_2Cl_2 (100 mL) was added and washed with H_2O (2 times, 50 mL). The organic phase was dried, and evaporated and the residue was purified by flash chromatography (silica gel, 30% EtOAc in Hexane) to give primarily the 1 substituted product (1.7015 g, 28% yield).



(Agonist)

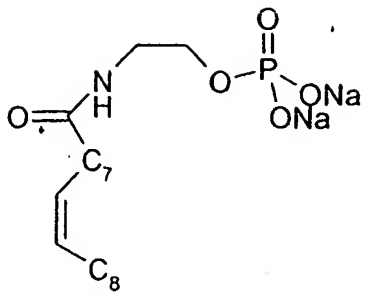
$P(O)Cl_3$ (0.259 mL) was added to a mixture of 1 and 2 substituted 1,2,3-cyclohexanetriol oleate (1 g), EtOAc (20 mL) and Et_3N (1.75 mL). The mixture was stirred at 60 C for 2 h. The mixture was cooled and the Et_3NHCl was filtered off. NaOH (0.2 M, 40 mL) was added and the mixture was stirred for 2 h. EtOAc (100 mL) was added and the organic layer was washed with H_2O (2 times, 30 mL). The organic layer was evaporated to give the product.

8.



(Intermediate)

Oleoyl chloride (7.69 mL) was added to a solution of ethanolamine (2.81 mL), THF (30 mL), $CHCl_3$ (150 mL), and Et_3N (16.2 mL). The mixture was stirred at room temperature for 12 h. $CHCl_3$ (100 mL) was added and washed with H_2O (2 times, 50 mL). The organic phase was dried, and evaporated and the residue was purified by flash chromatography (silica gel, 10% to 50 EtOAc in Hexane) to give the product (several grams).



(Agonist)

$P(O)Cl_3$ (0.472 mL) was added to a mixture of 2-oleoyl 1-ethanolamide (1.5 g), EtOAc (35 mL), and Et_3N (3.21 mL). The mixture was stirred at 60 C for 1.25 h and then the mixture was cooled and the triethylamine hydrochloride was filtered off. Sodium hydroxide (45 mL of 0.2 M) was added and the biphasic solution was stirred for 12 h. EtOAc (200 mL) was added along with brine (200 mL) and the layers were separated and the organic layer was evaporated and pumped to dryness. Thin Layer chromatography (silica gel, $CHCl_3$ 60, MeOH 30, HOAc 1, H_2O 1) of the product was compared to LPA (Sigma). The product ran with an Rf of ~0.05 and the LPA (Sigma) ran at 0.1.

PRIMARY SCHWANN CELL CULTURE

Sciatic nerves were excised from postnatal day 3 rat pups, and Schwann cells purified essentially as previously described[12]. Cells were grown on poly-L-lysine (0.1 mg/ml) coated dishes, or on poly-L-lysine and laminin (10 µg/ml) coated 8-well plastic Chamber Slides (Nunc) and glass coverslips. Growth medium was DMEM (Gibco) supplemented with 10% FCS, 20 µg/ml pituitary extract (Sigma), 2 µM forskolin, and penicillin/streptomycin. Schwann cell cultures were >98% pure as assessed by anti-P_0 and anti-S100 (Dako) immunofluorescence. For proliferation experiments, cultures at 24 h post-serum withdrawal were pulsed for an additional 24 h with BrdU, and cells were fixed and processed for BrdU immunofluorescence (BrdU Labeling and Detection Kit , Boehringer Mannheim).

NORTHERN BLOT ANALYSES

Total RNA was isolated from mouse sciatic nerves at various ages, and from cultured rat Schwann cells using Trizol reagent (Gibco). Northern blots of 7, 10, or 15 µg of RNA

were made using standard protocols, and were probed with ^{32}P-labelled open reading frame fragments of murine LP family receptor cDNAs at $5X10^6$ cpm/ml.

ISEL+ IDENTIFICATION OF APOPTOTIC CELLS

Schwann cells were grown to near-confluence on 8-well Chamber Slides. Media was changed to serum free DMEM, with or without LPA or other treatments as indicated. Cells were fixed in 4% paraformaldehyde 48 h later, and ISEL+ analyses were performed as previously described[18, 19]. All experimental treatments were performed at least 3 times in duplicate and over 1000 cells were counted per well. For counts of cell number, non-apoptotic DAPI-labeled nuclei (those with a normal , oval morphology and absence of any ISEL+ labeling) were counted in 10 fields of view (at 400X) per well. Cell counts were performed on duplicate slide wells from at least 3 experiments per condition.

AKT WESTERN BLOT ANALYSES

Schwann cells were grown to confluence in 6-well dishes and switched to DMEM/1% FCS for 24 h to reduce basal Akt phosphorylation. Media was changed to serum-free DMEM with our without pharmacological inhibitors, and 2 h later LPA (1 μM) was added. Media was removed at the indicated times and cell samples prepared, western blotted, and detected using the PhosphoPlus Ser473 Akt Antibody kit (New England Biolabs).

TRANSFECTION

Schwann cells were grown on glass coverslips to approximately 80% confluency, and were transfected for 3 h using Lipofectamine Plus (Gibco) in DMEM/10% FCS (no antibiotic). pFLAG/VZG-1[3] contains the complete open-reading frame of murine *lp$_{A1}$/vzg-1* fused to an N-terminal FLAG epitope sequence in the plasmid pFLAG/CMV2 (Kodak/IBI). pFLAG/BAP control plasmid was obtained from Kodak/IBI. After transfection, cells were switched to growth medium for 12 h to allow for protein expression, and then switched to serum free DMEM with or without LPA for 24 h. Cells were fixed for 30 min with 4% paraformaldehyde, and sequentially processed for fluorescent ISEL according to manufacturer's instructions (Fluorescein In Situ Cell Death Kit, Boehringer Mannheim) and anti-FLAG immunofluorescence (anti-FLAG M2 monoclonal antibody, Kodak/IBI, 1:6000). Three separate experiments were performed in

triplicate and all transfected cells on each coverslip were counted (~150-350 cells/coverslip). Baseline apoptosis due to transfection alone was assessed in parallel coverslips maintained in 10% FCS (in which untransfected cells exhibit < 1% apoptosis), and this value was subtracted from each experimental value.

EXAMPLE 2

LPA AND PROMOTION OF SCHWANN CELL SURVIVAL

Schwann cells exhibit little cell death (< 1%) when cultured in standard growth medium containing fetal calf serum (FCS; data not shown, [8]). However, apoptosis can be initiated in primary cultures of Schwann cells by withdrawal of serum[8], which serum includes survival factors likely supplied by growing axons *in vivo*.

To examine the possible survival-promoting effects of LPA, apoptosis was induced by switching Schwann cells to serum-free medium. In control cultures, cell death began soon after serum withdrawal, and by 48 h, significant cell loss was observed, with many cells exhibiting an apoptotic morphology (Fig. 2a). Treatment of Schwann cells with LPA over 48 h led to a clear reduction (of up to 60%; see Fig. 2b) in the number of apoptotic cells, as identified both morphologically and after fixation and staining using ISEL+ (In Situ End-Labeling +)[18, 19], a technique that labels the fragmented DNA ends that are an apoptotic hallmark[20] (Fig. 2a). LPA significantly reduced SC apoptosis, assessed using ISEL+, at doses as low as 10 nM (Fig. 2b), suggesting the activation of a high-affinity receptor(s) such as LP_{A1}/VZG-1, which continues to be expressed following serum withdrawal (Fig. 2b, inset). LPA treatment did not increase the number of Schwann cells incorporating bromodeoxyuridine (BrdU; Fig. 2a, c), demonstrating that the dose-dependent increase in cell number in LPA-treated cultures (Fig. 2d) was due to increased cell survival, and not to proliferation.

There is prominent expression of at least one of the S!P receptors [4, 17], LP_{B3}, in neonatal Schwann cells *in vitro* (Fig. 1). However, treatment with S1P, which has been reported to act (albeit *intracellularly*) to promote cell survival [21], did not reduce SC apoptosis (Fig. 2e). These data indicate that the survival effect is LPA-specific and that

there are different functions for these related lipid mediators and their GPCRs in Schwann cells.

EXAMPLE 3

LPA AND NEUREGULINS

The efficacy of LPA in promoting survival was further compared to that of a family of proven Schwann cell survival factors, the neuregulins[8, 9] (NRGs, also referred to as ARIA[22], NDF [7], or GGF[23]). A single NRG gene encodes a group of alternatively-spliced protein factors that signal through the receptor tyrosine kinases Erb2, Erb3, and Erb4[24]. LPA (1 μM) was as effective in promoting Schwann cellsurvival as a maximal dose (100 ng/ml) of a truncated NRG-isoform encompassing the EGF-like domain [25] that was previously shown to be highly effective in promoting survival[8] (Fig. 2d, f). LPA and NRG β did not have a significantly synergistic effect when added together at maximal (Fig. 2f) or half-maximal (not shown) doses, suggesting that their distinct receptors and signalling pathways may converge on common downstream effectors.

Although the lipid nature of LPA makes it experimentally difficult to assess whether it is produced by peripheral nerve axons, as are NRGs[7, 24], like the NRGs, LPA can exist in both membrane-bound and soluble forms. LPA may be released by Schwann cells themselves as an autocrine product of lipid metabolism during the elaboration of the myelin sheath, which is about 80% lipid, including complex phospholipids[13]. Moreover, as LPA is released by activated platelets[14], it is likely to be present following peripheral nerve injury, where it could influence regeneration by promoting the survival of Schwann cells.

EXAMPLE 4

LPA RECEPTOR ACTIVATION

Prevention of Schwann cell apoptosis by LPA was inhibited by pretreatment of Schwann cells with pertussis toxin (PTX; Fig 3a), indicating the involvement of G_i, to which LP_{A2}/VZG-1/edg 2 has been demonstrated to couple directly[3]. Accordingly, the activation of the LPA receptors, including LP_{A2}/VZG-1/edg 2, are believed to be directly involved in the promotion of Schwann cellsurvival.

EXAMPLE 5

P13K/AKT PATHWAY

LPA-dependent survival was also blocked completely by two PI3K inhibitors, wortmannin and LY 294002 (Fig. 3a). Sequential activation of PI3K and the serine-threonine kinase Akt (PKB) has been linked to the prevention of apoptosis in various cell types through phosphorylation of BAD, a pro-cell death member of the Bcl-2 family. As NRG can also activate a PI3K pathway [28], these data could explain the lack of a synergistic survival effect of LPA and NRG β (see above, Fig. 2f). Addition of 1 μM LPA to Schwann cells in serum-free medium induced a rapid and transient increase in the phosphorylation of Akt at a site (Ser473) required for its activation[11], as detected by western blot analysis (Fig. 3b). This accumulation of phospho-Akt in response to LPA was dependent on PI3K, as it was blocked by both wortmannin and LY294002, but not by the MAP kinase pathway inhibitor PD 98059 (Fig. 3c). These data identify LPA as a novel activator of the PI3K/Akt pathway, and implicate the activity of this pathway in promoting Schwann cell survival.

EXAMPLE 6

OVEREXPRESSION OF LP_{A1} RECEPTOR

The prominent expression of *lp_{A1}/vzg-1* in the absence of another reported LPA receptor gene[4, 29] (Fig. 1), along with the effectiveness of LPA at low nanomolar doses indicates that this high affinity receptor mediates LPA-dependent Schwann cell survival. In addition, FLAG epitope-tagged LP_{A1}/VZG-1 was overexpressed by transfection with an expression construct to determine if this receptor did mediate LPA-dependent Schwann cell survival. After overexpressing FLAG epitope-tagged LP_{A1}/VZG-1 by transfecting with an expression vector and withdrawing the serum, the apoptotic transfected cells were identified 24h later by double-labelling for anti-FLAG immunofluorescence and fluorescent ISEL (Fig. 4a). Overexpression of epitope-tagged LP_{A1}/VZG-1 significantly potentiated Schwann cell survival in serum-free medium (compared to transfection with a FLAG-tagged bacterial alkaline phosphatase (BAP) control construct), both with and without a sub-maximal (0.1 μM) dose of LPA (Fig. 4b). LP_{A1}/VZG-1 overexpression also modestly potentiated the effect of 1μM LPA (Fig. 4b); the smaller effect likely reflects

maximal activation of endogenous receptors at this dose. The increase in survival of LP_{A1}/VZG-1-transfected cells even in the absence of added LPA may reflect basal receptor coupling activity, which has been observed with overexpression of GPCRs[3, 30], and/or residual effects of serum-derived LPA present in the media prior to 24 h serum withdrawal (see Methods). These results, together with the LPA potency and expression data noted above, indicates that prevention of Schwann cell apoptosis by LPA can be mediated by the high affinity LPA receptor LP_{A1}/VZG-1.

EXAMPLE 7

Null mice for LP_{A1} /VZG-1/ edg-2 were created. As discussed below, analysis of the null mice support the findings above that the LP_{A1} /VZG-1/ edg-2 receptor and its activation with agonists, promotes survival of myelin producing cells.

As expected, the null mice showed no expression of the LP_{A1} /VZG-1/ edg-2 gene either in the sciatic nerves in 7 day postnatal mice or in neonatal Schwann cell cultures. (see Fig. 5c)

An analysis of the Schwann cells in the adult null mice using staining for apoptotic DNA fragmentation using ISEL+ or nuclei staining using DAPI clearly exemplifies that there is a decrease in Schwann cell survival in the null mice. This indicates that without the edg-2 receptor (and activation thereof –through serum-based LPA or through other agonists) myelin producing cells are subject to increased apoptosis. (See Figures 5d and 5e) Similarly examination of the sciatic nerves in the null mice showed characteristics of apoptosis, namely nuclear chromatin condensation and cytoplasmic fragmentation. (See Fig. 5f)

Furthermore, using the null mice, it was shown that Schwann cells isolated from the sciatic nerves of the null mice compared to the wild-type mice showed no process retraction and cell spreading in response to 100 nM LPA, (see Fig. 5a and 5b) In wild-type cells, process retraction and cell spreading occurs in response to nM concentrations of LPA.

Hence, based on the above analysis, the null mice exhibited a decrease in Schwann cell survival *in vivo* and an inability to respond normally to LPA further exemplifying that the activation of the LP_{A1}/VZG-1/ edg-2 receptor promotes survival of Schwann cells.

It will be well understood by a skilled person in the art that the invention as herein described and exemplified may be modified without departing from the scope of the invention as defined in the claims appended hereto.

Incorporated herein by reference are the disclosures of the following articles, some of which are identified with footnotes hereinabove:

1. Moolenaar, W.H., Kranenburg, O., Postma, F.R. & Zondag, G.C.M. Lysophosphatidic acid: G-protein signalling and cellular responses. *Current Opinion in Cell Biology* **9**, 168-173 (1997).

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5. Syroid, D.E., *et al.* Cell death in the Schwann cell lineage and its regulation by neuregulin. *Proc Natl Acad Sci U S A* **93**, 9229-34 (1996).

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18. Vartanian, T., Corfas, G., Li, Y., Fischbach, G.D. & Stefansson, K. A role for the acetylcholine receptor-inducing protein ARIA in oligodendrocyte development. *Proc Natl Acad Sci U S A* **91**, 11626-30 (1994).

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WE CLAIM:

1. A method for promoting survival of myelin producing cells comprising the step of treating myelin producing cells with a survival promoting amount of an LPA receptor agonist.
2. A method according to claim 1, wherein said myelin producing cells are oligodendrocytes.
3. A method according to claim 1, wherein said myelin producing cells are Schwann cells.
4. The method according to claim 3, wherein the LPA receptor agonist is an LP_{A1}/VZG-1/edg-2 receptor agonist.
5. The method according to claim 3, wherein the LPA receptor agonist is LPA.
6. The method according to claim 3, wherein the Schwann cell is treated in vitro, or ex vivo.
7. The method according to claim 6 further comprising the step of culturing the Schwann cell in a serum free culture growth medium comprising said LPA receptor agonist.
8. The method according to claim 7 wherein said LPA receptor agonist is an LP_{A1}/VZG-1/edg-2 receptor agonist.
9. A method for enhancing the development or regeneration of myelin by promoting the survival of myelin producing cells according to claim 1.
10. The method according to claim 9 wherein said myelin producing cells are oligodendrocytes.
11. The method according to claim 9 wherein said myelin producing cells are Schwann cells.
12. The method according to claim 11 wherein said LPA receptor agonist is an LP_{A1}/VZG-1/edg-2 receptor agonist.
13. A method for treating a subject to promote survival of endogenous myelin producing cells, comprising the step of delivering to the subject an effective amount of an LPA receptor agonist.
14. A method according to claim 9 wherein said endogenous myelin producing cells are endogenous Schwann cells.
15. A method according to claim 9 wherein said endogenous myelin producing cells are endogenous oligodendrocytes.

16. A method for treating a subject suffering from a neurological disorder involving a loss of myelination comprising the step of delivering to the subject an effective amount of an LPA receptor agonist.
17. A method according to claim 12 wherein said neurological disorder is Multiple Sclerosis.
18. A serum-free medium useful to culture myelin producing cells comprising a supplementary LPA receptor agonist in an amount effective to promote survival of said myelin producing cells.
19. A serum-free medium according to claim 14 wherein said myelin producing cells are Schwann cells.
20. A serum-free medium according to claim 14 wherein said myelin producing cells are oligodendrocytes.

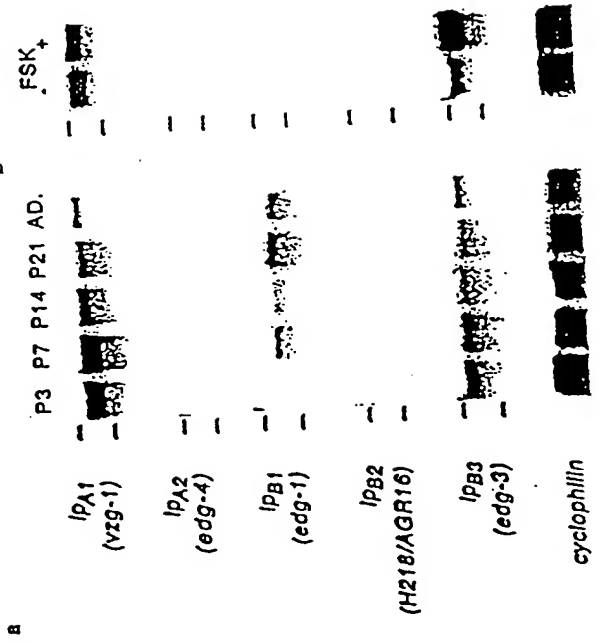

a
b
P3 P7 P14 P21 AD.
FSK
lp_{A1} (vzg-1)
lp_{A2} (edg-4)
lp_{B1} (edg-1)
lp_{B2} (H218/AGR16)
lp_{B3} (edg-3)
cyclophilin

Figure 1

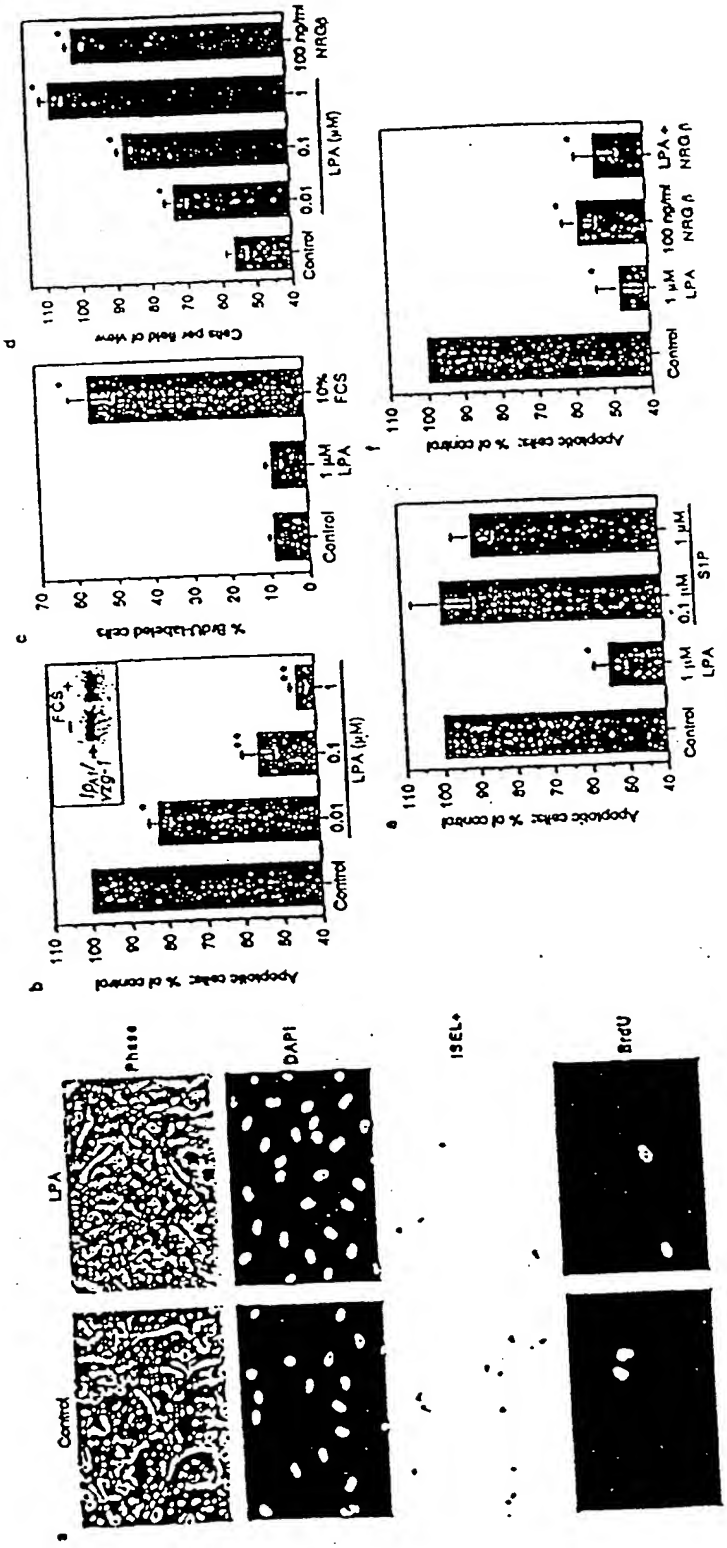

a
Control
LPA
Phase
DAPI
TUNEL
BrdU
b
Apoptotic cells: % of control
Control
0.01
0.1
1
LPA (μM)
FCS
c
% BrdU-labeled cells
Control
1 μM LPA
10% FCS
d
Cells per field of view
Control
0.01
0.1
1
LPA (μM)
100 ng/ml NRG-β
e
Apoptotic cells: % of control
Control
1 μM LPA
0.1 nM
1 μM
S1P
f
Apoptotic cells: % of control
Control
1 μM LPA
100 ng/ml NRG-β
LPA + NRG-β

Figure 2

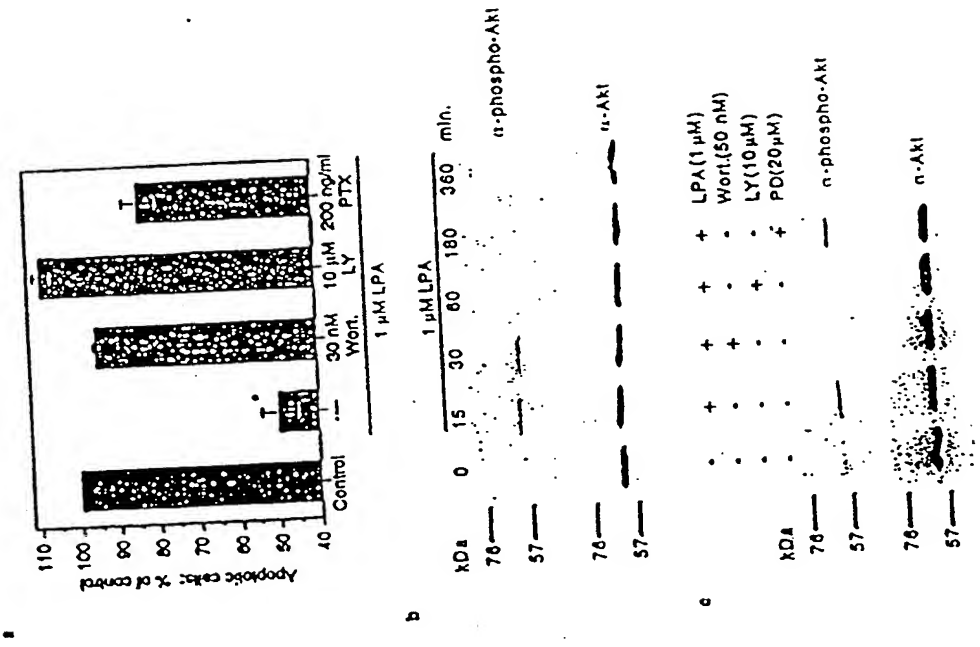

a
Apoptotic cells: % of control
110
100
90
80
70
60
50
40
Control
30 nM Wort.
10 μM LY
200 ng/ml PTX
1 μM LPA
b
1 μM LPA
0
15
30
60
180
360
min.
kDa
78
57
α-phospho-Akt
α-Akt
c
LPA (1 μM)
Wort.(50 nM)
LY(10 μM)
PD(20μM)
kDa
78
57
α-phospho-Akt
α-Akt

Figure 3

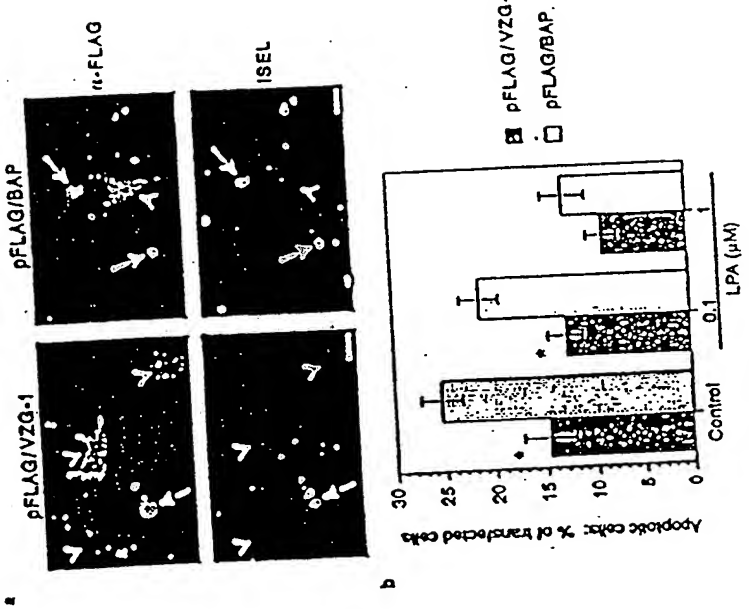
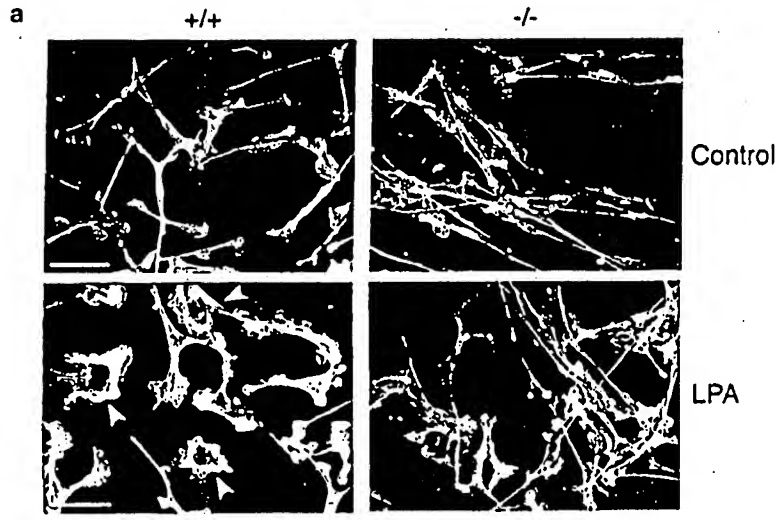
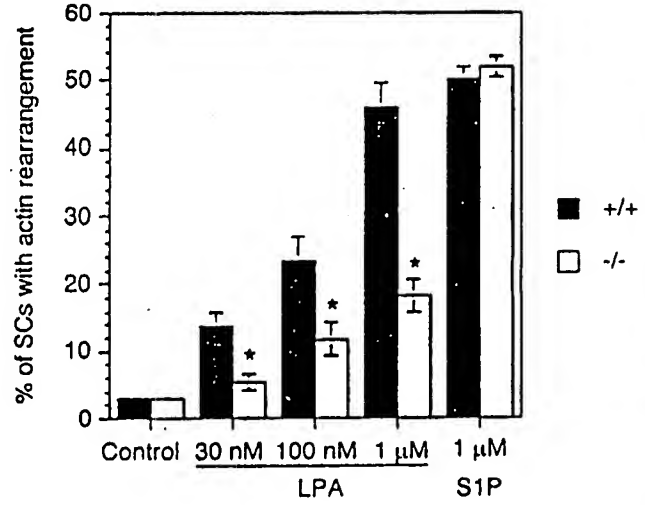
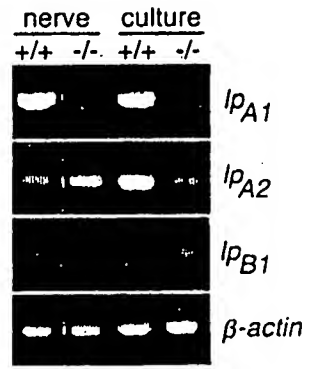
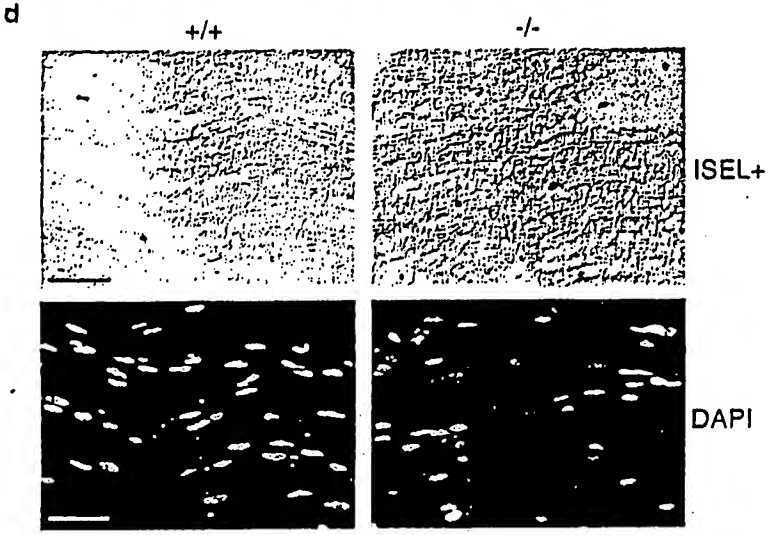

pFLAG/BAP
pFLAG/VZG-1
α-FLAG
ISEL
pFLAG/VZG-1
pFLAG/BAP
Control
0.1
1
LPA (μM)
Apoptotic cells: % of transfected cells
30
25
20
15
10
5
0

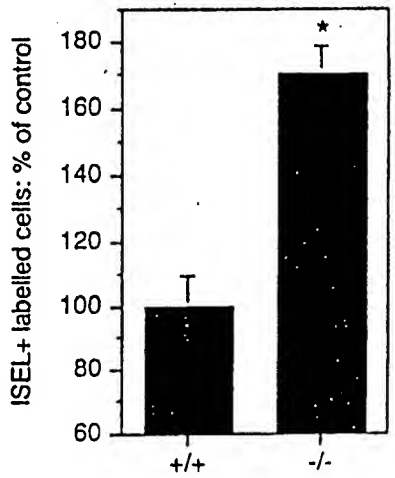
Figure 4


a
+/+
-/-
Control
LPA


b
% of SCs with actin rearrangement
60
50
40
30
20
10
0
Control
30 nM
100 nM
1 μM
1 μM
LPA
S1P
+/+
-/-


c
nerve
culture
+/+
-/-
+/+
-/-
lpA1
lpA2
lpB1
β-actin


d
+/+
-/-
ISEL+
DAPI


e
ISEL+ labelled cells: % of control
180
160
140
120
100
80
60
+/+
-/-

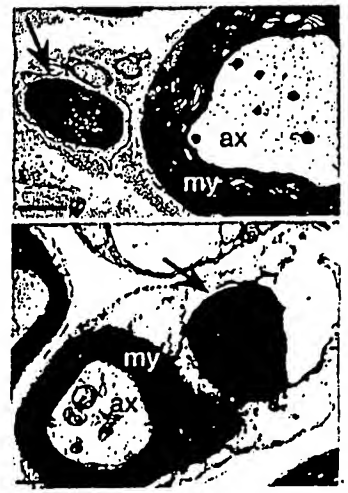

f
ax
my
my
ax

Figure 5